FOOD STANDARDS
Australia New Zealand
Te Mana Kounga Kai – Ahitereiria me Aotearoa

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308-24

Supporting document 1

Safety assessment – Application A1303

Food derived from herbicide-tolerant sugar beet line KWS20-1

Executive summary

Background

Application A1303 seeks approval for the sale and use of food derived from sugar beet line KWS20-1 that has been genetically modified (GM) for tolerance to the herbicides dicamba, glufosinate and glyphosate.

Tolerance to these herbicides in KWS20-1 is conferred thorough expression of the:

- *dmo* gene from *Stenotrophomonas maltophilia*, which encodes a dicamba mono-oxygenase (DMO) protein and provides tolerance to dicamba;
- *pat* gene from *Streptomyces viridochromogenes*, which encodes a phosphinothricin-*N*-acetyltransferase (PAT) protein and provides tolerance to glufosinate;
- *cp4 epsps* gene from *Agrobacterium* sp. strain CP4, which encodes a 5-enolpyruvylshikimate-3-phosphate synthase protein (CP4 EPSPS) and provides tolerance to glyphosate.

Food Standards Australia New Zealand (FSANZ) has previously assessed the DMO, PAT and CP4 EPSPS proteins.

This safety assessment addresses food safety and nutritional issues associated with the GM food. It therefore does not address:

- risks related to the environmental release of GM plants used in food production
- risks to animals that may consume feed derived from GM plants
- the safety of food derived from the non-GM (conventional) plant.

History of use

Sugar beet has a long history of safe use in the food supply. The major food product derived from sugar beet is refined sugar (sucrose), which is consumed in large quantities worldwide. The by-products of sugar beet processing – molasses and pulp – are primarily used as animal feed.

Molecular characterisation

The genes encoding DMO (*dmo*), PAT (*pat*) and CP4 EPSPS (*cp4 epsps*) were introduced into sugar beet line KWS20-1 via *Agrobacterium*-mediated transformation. Detailed molecular

analyses indicate a single copy of each of the three gene cassettes is present at a single insertion site in the KWS20-1 genome. There are no extraneous plasmid sequences or antibiotic resistance genes present in this line.

The introduced genetic elements were shown by molecular techniques and phenotypic analyses to be present within a single locus and stably inherited across multiple generations.

Characterisation and safety assessment of new substances

All three novel proteins (DMO, PAT and CP4 EPSPS) are expressed throughout KWS20-1 (except PAT which was not detected in harvestable root tissue). Expression levels for DMO and CP4 EPSPS were also lowest in harvestable root.

Characterisation studies confirmed that the PAT and CP4 EPSPS proteins are identical to proteins previously assessed by FSANZ, while the DMO protein expressed in KWS20-1 is highly similar to DMO proteins previously assessed by FSANZ. All proteins are rapidly degraded and heat inactivated, based on studies submitted with this application and/or conclusions from previous assessments. Updated bioinformatics studies for all three proteins confirmed the lack of any significant amino acid sequence similarity to known protein toxins or allergens. Taken together, the evidence supports the conclusion that DMO, PAT and CP4 EPSPS are not toxic or allergenic to humans.

Compositional analyses

Detailed compositional analyses were performed on KWS20-1. Statistically significant differences were found between KWS20-1 and the non-GM control for 8 of the 28 analytes evaluated in root, however these differences were all within the range established for existing commercial non-GM sugar beet varieties. Overall, the compositional data support the conclusion that there are no biologically significant differences in the levels of key constituents in root from KWS20-1 compared to non-GM sugar beet varieties available on the market.

Conclusion

No potential public health and safety concerns have been identified in the assessment of herbicide-tolerant sugar beet line KWS20-1. On the basis of the data provided in the present application and other available information, food derived from KWS20-1 is considered to be as safe for human consumption as food derived from conventional non-GM sugar beet varieties.

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List of Abbreviations

Abbreviation	Description
AFSI	Agriculture and Food Systems Institute
BLOSUM	BLOcks SUbstitution Matrix
bp	base pair
COMPARE	COMprehensive Protein Allergen Resource
CTP	chloroplast transit peptide
DMO	dicamba mono-oxygenase
DNA	deoxyribonucleic acid
dw	dry weight
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
FASTA	fast alignment search tool – all
FAO	Food and Agriculture Organisation of the United Nations
FSANZ	Food Standards Australia New Zealand
g	gram
gDNA	genomic DNA
GM	genetically modified
KASP	kompetitive allele specific PCR
kDa	kilodalton
LB	left border
LOQ	limit of quantitation
MT	million tons
NCBI	National Centre for Biotechnology Information
nt	nucleotide
OECD	Organisation for Economic Co-operation and Development
OGTR	Office of the Gene Technology Regulator
PAT	phosphinothricin-*N*-acetyltransferase
PCR	polymerase chain reaction
RB	right border
RF	reading frame
RNA	ribonucleic acid
T-DNA	transfer DNA
µg	microgram
USDA	United States Department of Agriculture
UTR	untranslated region

1 Introduction

Food Standards Australia New Zealand (FSANZ) received an application from Bayer CropScience Proprietary Limited to vary Schedule 26 in the Australia New Zealand Food Standards Code. The variation is to include food from a new genetically modified (GM) sugar beet line KWS20-1, with the OECD Unique Identifier KB-KWS20Ø1-6. This sugar beet line is tolerant to the herbicides dicamba, glufosinate and glyphosate.

- Tolerance to dicamba is achieved with the expression of the dicamba mono-oxygenase (DMO) protein, encoded by the *dmo* gene from the bacterium *Stenotrophomonas maltophilia*. The DMO protein has been assessed by FSANZ in 6 previous applications.[1]
- Tolerance to glufosinate is achieved with the expression of the phosphinothricin-*N*-acetyltransferase (PAT) protein, encoded by the *pat* gene from the bacterium *Streptomyces viridochromogenes*. The PAT protein has been assessed by FSANZ in numerous previous applications.
- Tolerance to glyphosate is achieved with the expression of the CP4 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) protein, encoded by the *cp4 epsps* gene from the bacterium *Agrobacterium* sp. strain CP4. The CP4 EPSPS protein has been assessed by FSANZ in a number of previous applications.

If approved, food derived from sugar beet line KWS20-1 may enter the Australian and New Zealand food supply as imported food products.

2 History of use

2.1 Host organism

The host organism is sugar beet (*Beta vulgaris* L. ssp. *vulgaris*). The conventional sugar beet line 04E05B1DH05 was used as the parental variety for the genetic modification described in this application.

Sugar beet has a long history of safe use in the food supply. Sugar beet has been cultivated as a source of sugar since the late 1700s (OECD 2001; FAO 2009). Beet sugar now accounts for ~30% of the world's sucrose production, with the remainder derived from sugarcane (Dohm et al. 2014). Global production of sugar beet was 270 MT[2] in 2022 (FAOSTAT 2024). The leading producer of beet sugar in the world is the European Union, which produced 14.8 MT in 2023/24 (USDA 2024). No sugar beet is grown commercially for sugar production in Australia or New Zealand (FAOSTAT 2024).

The Australian sugar industry produces sugar from sugarcane, with more than 80% of all sugar produced in Australia being exported as raw sugar.[3] New Zealand relies on imported raw sugar, primarily from Australia, for its domestic sugar production.[4] Neither Australia nor New Zealand import significant quantities of unprocessed sugar beet (FAOSTAT 2024).

Sugar beet roots in their unprocessed form are rarely used as food or feed (OECD 2002). The major food product derived from sugar beet is refined sugar (sucrose), which is used as a

[1] Applications A1063 (MON87798 soybean); A1080 (MON88701 cotton); A1118 (MON87419 corn); A1192 (MON87429 corn); A1216 (MON94100 canola); A1276 (MON94313 soybean)
[2] million tons
[3] Department of Agriculture, Fisheries and Forestry (DAFF) – Sugar: https://www.agriculture.gov.au/agriculture-land/farm-food-drought/crops/sugar#sugar-industry-assistance-and-reform
[4] World Bank – New Zealand imports of raw cane sugar 2023: https://wits.worldbank.org/trade/comtrade/en/country/NZL/year/2023/tradeflow/Imports/partner/ALL/product/170111

sweetener and in a variety of processed food products. The by-products of sugar processing – molasses and pulp – are primarily used as animal feed, though molasses does have minor uses in food (OECD 2002). The leaves and tops of sugar beets are also used as animal feed.

2.2 Donor organisms

2.2.1 Stenotrophomonas maltophilia

The *dmo* gene is derived from the Gram-negative bacterium *Stenotrophomonas maltophilia* strain DI-6, isolated from soil at a dicamba manufacturing plant (Krueger et al. 1989). *S. maltophilia* is ubiquitously distributed in the environment, particularly the rhizosphere (Ryan et al. 2009). It has also been isolated from a number of foods, including leafy vegetables (Li et al. 2019) and cheese (Todaro et al. 2011; Okuno et al. 2018). *S. maltophilia* is considered an emerging human pathogen, and can cause severe disease in susceptible populations under favourable conditions (Mukherjee and Roy 2016; Brooke 2021). However, it has low virulence and community-acquired infections are rare (Lira et al. 2017).

The *dmo* gene in KWS20-1 has been manipulated through standard DNA cloning methods subsequent to its isolation, meaning that extraneous material from *S. maltophilia* would not have been transferred to KWS20-1.

S. maltophilia has a history of safe use as the donor organism of the *dmo* gene and been assessed in several previous applications to FSANZ.[5]

2.2.2 Streptomyces viridochromogenes

The source of the *pat* gene is the Gram-positive bacterium *Streptomyces viridochromogenes* (Wohlleben et al. 1988). *S. viridochromogenes* is widespread in the environment and is not pathogenic to humans or animals. The *pat* gene produces a protein that is structurally and functionally equivalent to the protein encoded by the *bar* gene from the closely related species *S. hygroscopicus* (Wehrmann et al. 1996). While *S. viridochromogenes* and *S. hygroscopicus* themselves do not have a history of use in food, the *pat* and *bar* genes have been used to confer glufosinate tolerance in food-producing crops for almost three decades with no toxicity or allergenicity concerns (ILSI 2016).

2.2.3 Agrobacterium sp. strain CP4

The *cp4 epsps* gene is derived from *Agrobacterium* sp. strain CP4 (Padgette et al. 1996). *Agrobacterium* species are known soil-borne plant pathogens but are not pathogenic to humans or other animals.

This bacterium has been assessed by FSANZ as the donor organism of the *cp4 epsps* gene in 16 previous GM applications in a range of crops, including sugar beet.[6]

2.2.4 Other organisms

Genetic elements from several other organisms have been used in the genetic modification of KWS20-1 (see Table 1 and Appendix 1). These genetic elements are non-coding sequences and are used to regulate the expression of the *dmo*, *pat* and *cp4 epsps* genes.

[5] Applications A1063 (MON87798 soybean); A1080 (MON88701 cotton); A1118 (MON87419 corn); A1192 (MON87429 corn); A1216 (MON94100 canola); A1276 (MON94313 soybean)
[6] Applications A338 (GTS 40-3-2 soybean), A355 (1445 cotton), A363 (GT73 canola), A378 (GTSB77 sugar beet), A383 (New Leaf Plus potatoes), A416 (NK603 corn), A525 (H7-1 sugar beet), A548 (MON88017 corn), A553 (MON88913 cotton), A575 (J101 and J163 lucerne), A592 (MON89788 soybean), A1049 (MON87705 soybean), A1066 (MON87427 corn), A1071 (MON88302 corn), A1097 (MON87411 corn), and A1192 (MON87429 corn)

3 Molecular characterisation

Molecular characterisation is necessary to provide an understanding of the genetic material introduced into the host genome and helps to frame the subsequent parts of the safety assessment. The molecular characterisation addresses three main aspects:

- the transformation method together with a detailed description of the DNA sequences introduced to the host genome
- a characterisation of the inserted DNA, including any rearrangements that may have occurred as a consequence of the transformation
- the genetic stability of the inserted DNA and any accompanying expressed traits.

3.1 Transformation method

To create sugar beet line KWS20-1, conventional sugar beet line 04E05B1DH05 was transformed using the plasmid PV-BVHT527462 (Figure 1). The transformation and subsequent development steps for KWS20-1 are outlined in the flowchart in Appendix 2 and summarised below.

Transformation of the 04E05B1DH05 line was achieved by co-culturing shoot segment tissues excised from the embryos of germinated conventional seed with *Agrobacterium tumefaciens* containing the PV-BVHT527462 plasmid (Lindsay and Gallois 1990). The transformed calli were placed on selection medium containing DL-phosphothricin (PPT) and timentin. PPT inhibits the growth of untransformed cells and timentin inhibits *Agrobacterium* overgrowth. The calli were then transferred to media to encourage shoot and root development. Rooted plants with normal phenotypes were screened to identify plants carrying a single copy of the transfer DNA (T-DNA), no vector backbone, and no insertion into repetitive regions or gene sequences. These plants were selected and transferred to soil for growth and further assessment.

A single plant generated by this process (T0) was self-pollinated, producing T1 seed. T1 plants were screened using Kompetitive Allele Specific PCR (KASP) (Semagn et al. 2014) and Southern blotting to identify T1 plants carrying T-DNA but not the vector backbone. Twelve T1 plants that were homozygous for the T-DNA were crossed to produce T2 seed.

Subsequent generations were further evaluated for insert integrity, trait efficacy, phenotypic characteristics and agronomic performance. Sugar beet line KWS20-1 was then selected.

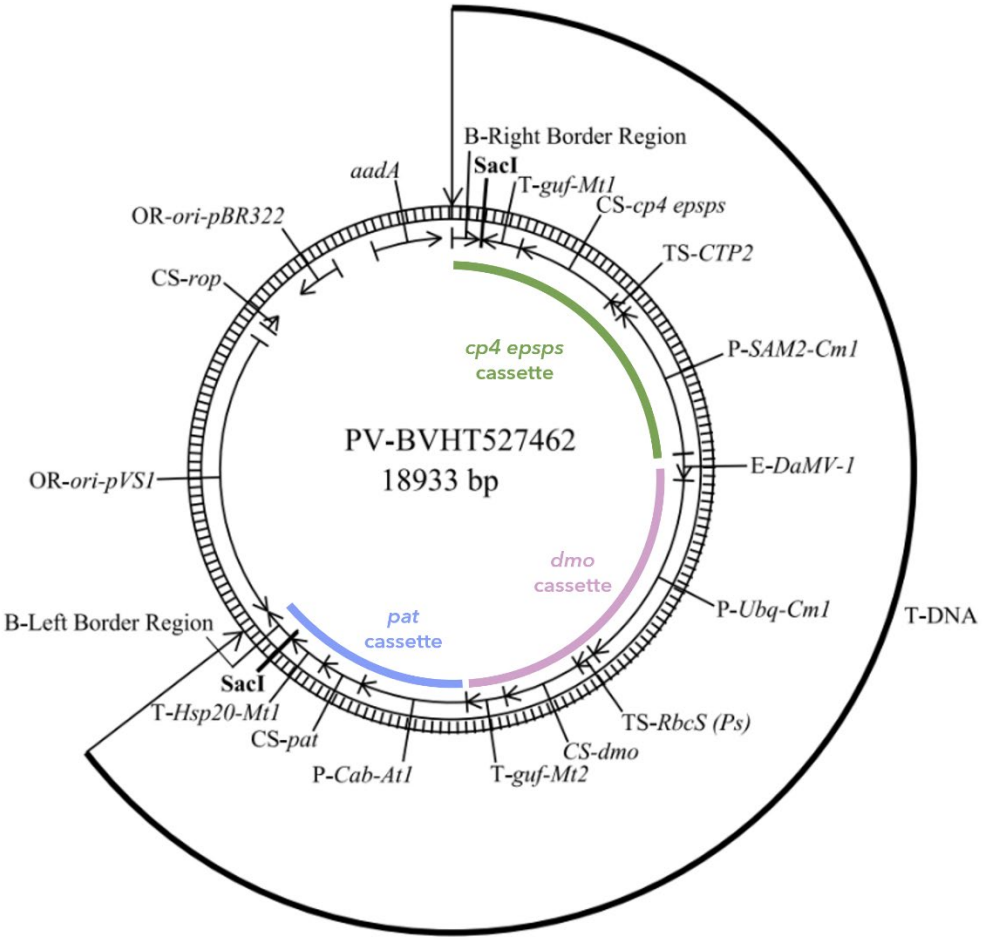

Figure 1. *Map of plasmid PV-BVHT527462. The T-DNA region between the left and right border regions was inserted into the sugar beet genome. This region contains the cp4 epsps, dmo and pat expression cassettes, as indicated.*

3.2 Detailed description of inserted DNA

Sugar beet line KWS20-1 contains T-DNA from the PV-BVHT527462 plasmid (Figure 1) and includes the *dmo*, *pat* and *cp4 epsps* expression cassettes. The final insert was 11,722 bp long (Figure 2).

Information on the genetic elements in the T-DNA used for transformation is summarised in Table 1. Additional detail, including intervening sequences used to assist with cloning, mapping and sequence analysis, can be found in Appendix 1.

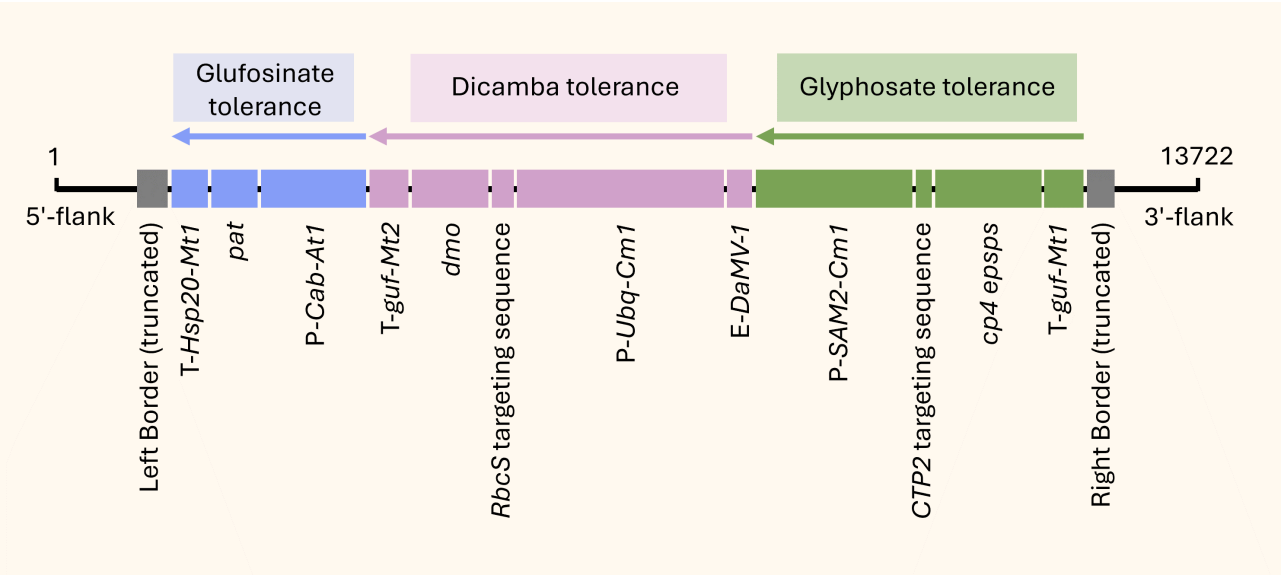

Figure 2. *Schematic of the inserted DNA in KWS20-1. The 11,722 bp insert, containing the pat, dmo and cp4 epsps expression cassettes, as well as 1000 bp of 5' and 3' flanking DNA, are shown.*

Table 1. Expression cassettes contained in the T-DNA of PV-BVHT527462

	Promoter	Enhancer/ Regulatory sequence	Coding sequence	Terminator	Notes
***dmo* cassette**	Promoter, leader and intron for a putative ubiquitin protein gene from *Cucumis melo*	Enhancer from a Dalia Mosaic Virus (DaMV) promoter region Targeting sequence and the first 27 amino acids from *Pisum sativum* (pea) rbcs gene family	Codon-optimised coding sequence for the dicamba mono-oxygenase (DMO) protein from *Stenotrophomonas maltophilia*	3' UTR from an expressed gene of unknown function from *Medicgo truncatula*	Confers dicamba tolerance
***pat* cassette**	Promoter and leader from a chlorophyll a/b-binding (CAB) protein from *Arabidopsis thaliana*	None	Codon-optimised coding sequence for the phosphinothricin *N*-acetyltransferase (PAT) protein from *Streptomyces viridochromogenes*	3' UTR from a putative *Hsp20* gene from *M. truncatula*	Confers glufosinate tolerance
***cp4 epsps* cassette**	Intron, 5' UTR, and promoter from a *SAM2* gene from *C. melo*	Targeting sequence of the *shkG* gene from *A. thaliana*	Codon-optimised *aroA* gene from *Agrobacterium tumefaciens* sp. strain CP4	3' UTR from an expressed gene of unknown function from *M. truncatula*	Confers glyphosate tolerance

3.3 Development of the sugar beet line from the original transformation

A breeding programme was undertaken for the purposes of:

- obtaining generations suitable for analysing the characteristics of KWS20-1
- ensuring that the KWS20-1 event is incorporated into elite lines for commercialisation.

A breeding history diagram depicting how KWS20-1 was derived from the original transformant was provided in the application as Confidential Commercial Information (CCI). While the full details of CCI cannot be provided in this public report, FSANZ has given regard to this information in its

assessment.

Table 2 indicates the specific generations and comparators used in the various analyses of KWS20-1.

Table 2. KWS20-1 generations used for various analyses

Analysis	Section	Generation(s) used	Comparators
Number of integration sites	Section 3.4.1	T2	04E05B1DH05
Absence of backbone and other sequences	Section 3.4.2	T2	04E05B1DH05
Insert integrity and site of integration	Section 3.4.3	T2	04E05B1DH05
Genetic stability	Section 3.4.4.1	T1, T2, T3, T4, T5	04E05B1DH05
Mendelian inheritance	Section 3.4.4.2	BC0S1, BC1S1, BC2S1	N/A
Expression of phenotype over several generations	Section 3.4.4.2	T2, T3, T4	04E05B1DH05

3.4 Characterisation of the inserted DNA and site(s) of insertion

A range of analyses were undertaken to characterise the genetic modification in KSW20-1. These analyses focused on the nature and stability of the inserted DNA and whether any unintended re-arrangements or products may have occurred as a consequence of the transformation procedure.

3.4.1 Number of integration site(s)

Southern blot analysis was used to determine the number of inserted DNA sequences in KWS20-1. Leaf-derived genomic DNA (gDNA) from the homozygous T2 generation of KWS20-1 was extracted and digested with restriction enzymes (*Xba*I or *Eco*32I). After electrophoretic separation and blotting, the DNA was hybridised with two combinations of radiolabelled probes covering the expected T-DNA insert. Genomic DNA from the conventional sugar beet line 04E05B1DH05 was used as a negative control, and 04E05B1DH05 DNA spiked with plasmid PV-BVHT527462 served as a positive control to confirm probe hybridisation.

As expected, no hybridisation bands were observed for the negative control samples digested with either *Xba*I or *Eco*32I. KWS20-1 gDNA digested with *Xba*I or *Eco*32I produced bands with sizes consistent with those expected for a single copy of T-DNA inserted at a single location within the sugar beet genome.

3.4.2 Absence of backbone and other sequences

Southern blot analysis was performed as described in section 3.4.1, but using a set of hybridisation probes spanning the backbone sequence of the transformation plasmid PV-BVHT527462. No hybridisation bands were observed for KWS20-1 genomic DNA digested with either *Xba*I or *Eco*32I. The conventional control DNA (04E05B1DH05) also produced no hybridisation bands, as expected, while the positive controls containing either 1 or 0.1 genome equivalents of *Xba*I or *Eco*32I-digested PV-BVHT527462 plasmid mixed with conventional control DNA produced single hybridisation bands of the expected sizes. These results confirmed there was no detectable integration of transformation vector backbone sequences into KWS20-1.

3.4.3 Insert integrity and site of integration

Sanger sequencing was performed on multiple overlapping polymerase chain reaction (PCR) fragments covering the insert and flanking sugar beet genomic DNA sequences of KWS20-1. The sequencing results confirmed that the insertion is 11,722 bp long and the genetic elements in the

inserted T-DNA are intact and organised as expected, with the exception of small terminal truncations in both the right and left border regions. These truncations would not have a functional impact on the expression of the inserted *dmo*, *pat* or *cp4 epsps* cassettes.

To examine the T-DNA insertion site, PCR primers flanking the insertion site were used to amplify genomic DNA from KWS20-1 and from the conventional control 04E05B1DH05. Sequence comparison of the products from KWS20-1 and conventional control indicate a 7 bp deletion of sugar beet genomic DNA occurred during T-DNA integration. All other flanking sequences in KWS20-1 were identical to those in the conventional control. Such changes during T-DNA insertion are common during *Agrobacterium*-mediated plant transformation due to double-stranded break repair mechanisms (Salomon and Puchta 1998; Anderson et al. 2016) and would not affect the expression of the *dmo*, *pat* or *cp4 epsps* cassettes.

3.4.4 Stability of the genetic changes in sugar beet line KWS20-1

The concept of stability encompasses both the genetic and phenotypic stability of the introduced trait over a number of generations. Genetic stability refers to maintenance of the modification (as produced in the initial transformation event) over successive generations. Phenotypic stability refers to the expressed trait remaining unchanged over successive generations.

3.4.4.1 Genetic stability

Southern blot analysis was used to show the genetic stability of the inserted *dmo*, *pat* and *cp4 epsps* expression cassettes in KWS20-1. Leaf-derived genomic DNA from five generations of KWS20-1 (T1, T2, T3, T4, T5) was extracted, digested with either *Xba*I or *Eco*32I, and hybridised with radiolabelled probes spanning the T-DNA insert, as described in section 3.4.1. Positive and negative controls were as described in section 3.4.1.

Hybridisation of each probe to the digested genomic DNA from KWS20-1 showed an equivalent band fingerprint across all five generations. No unexpected bands were observed. The consistency of these results confirmed that the inserted DNA is maintained stably in sugar beet line KWS20-1.

3.4.4.2 Phenotypic stability

Mendelian inheritance

Since the inserted T-DNA resides at a single locus within the KWS20-1 genome, it would be expected to be inherited according to Mendelian principles. To confirm this, the zygosity of plants in the BC0S1 generation (produced by self-pollination of BC0 seed which is hemizygous for the T-DNA insert), was first determined by two co-dominant KASP assays, which distinguish between homozygous positive, hemizygous positive and homozygous negative (non-transgenic) plants. Segregation analysis was then conducted for the BC0S1 generation and two subsequent generations (BC1S1, BC2S1) using qualitative PCR to test for the presence or absence of T-DNA.

According to Mendelian inheritance principles, the predicted zygosity of the BC0S1 generation was 1:2:1 (homozygous positive: hemizygous positive: homozygous negative) and the segregation ratio in all generations was 3:1 (T-DNA present: T-DNA absent). A Pearson's chi-square (χ^2) analysis was conducted to compare the observed and expected segregation ratios across these generations. The results in Tables 3 and 4 demonstrate that no statistically significant ($p < 0.05$) deviation from the expected segregation ratios were observed for any of the generations. These data support the conclusion that the inserted DNA is present at a single locus in the KWS20-1 genome and is inherited predictably according to Mendelian inheritance principles.

Table 3. Segregation results in one generation of KWS20-1 based on zygosity of inserted T-DNA

Generation	Total plants	Predicted zygosity ratio	Observed number of plants (expected number)			Statistical analysis	
			Homozygous Positive	Hemizygous positive	Homozygous Negative	χ^2	*P value*
BC0S1	200	1:2:1	50 (50)	104 (100)	46 (50)	0.480	0.787

Table 4. Segregation results in three generations of KWS20-1 based on presence or absence of T-DNA insert

Generation	Total plants	Expected segregation ratio	Observed number of plants (expected number)		Statistical analysis	
			T-DNA positive	T-DNA negative	χ^2	*P value*
BC0S1	200	3:1	154 (150)	46 (50)	0.427	0.514
BC1S1	200	3:1	150 (150)	50 (50)	0.000	1.000
BC2S1	200	3:1	154 (150)	46 (50)	0.427	0.514

Expression of phenotype over several generations

The expression of the DMO, PAT and CP4 EPSPS proteins in three generations of KWS20-1 (T2, T3 and T4) was examined. Western blot analysis was conducted on leaf tissue from each generation, with leaf tissue from the conventional line 04E05B1DH05 used as a negative control. *Escherichia coli*-produced versions of the three proteins were used as positive controls. In all three breeding generations, the DMO, PAT and CP4 EPSPS proteins migrated indistinguishably from the corresponding positive controls on the same Western blot. Some faint higher molecular weight bands were observed for the KWS20-1 samples on the DMO and PAT blots. These were attributed to small populations of dimeric and/or trimeric KWS20-1-derived DMO and PAT, likely as an artifact of the assay conditions. None of the proteins were detected in the tissue from the conventional control. These data support the conclusion that the CP4 EPSPS, DMO and PAT proteins are stably expressed over multiple generations.

3.4.5 Reading frame analysis

A bioinformatic analysis of the KWS20-1 insert, as well as the flanking DNA regions, was undertaken to identify whether any novel reading frames (RFs) had been created in KWS20-1 as a result of the DNA insertion, and whether any putative peptides encoded by the identified RFs have the potential for allergenicity or toxicity.

Sequences spanning the 5′ and 3′ insert-flank junctions of KWS20-1 were translated *in silico* from stop codon to stop codon (TGA, TAG, TAA) in all six reading frames.[7] A total of 10 putative peptides of eight amino acids or greater in length from the insert-flank junctions were identified. In addition, the entire KSW20-1 insert sequence was translated in all six reading frames.

The 10 insert-flank junction peptides as well as the six translated insert reading frames were investigated further to determine whether their amino acid sequences showed similarity with known allergen and toxin peptide sequences in established databases. These analyses are theoretical only, as it is highly unlikely that any of the identified putative peptides would be expressed *in planta*.

[7] Evaluation of sequences stop-to-stop codon is a more conservative approach compared to the evaluation of start-to-stop codon sequences.

3.4.5.1 Bioinformatic analysis for potential allergenicity

The putative peptides identified above were compared to known allergenic proteins listed in the COMprehensive Protein Allergen REsource (COMPARE)[8] database, from the Health and Environmental Science Institute. At the date of the search, there were 2,463 sequences in the allergen database (AD_2022). Sequences were also compared to the GenBank all protein database (PRT_2022), downloaded from the National Centre for Biotechnology Information (NCBI)[9], which contained 184,933,782 sequences at the date of download.

Three types of analyses were performed for this comparison:

(a) Full length sequence search – a FASTA alignment using a BLOSUM50 scoring matrix, which identifies blocks of residues with at least 50% sequence identity. Only matches with E-scores of $\leq 1\times10^{-5}$ were considered.
(b) 80-mer sliding window search – a FASTA alignment was performed comparing all contiguous 80 amino acids to the database entries. Only matches of greater than 35% similarity over ≥ 80 amino acids were considered.
(c) 8-mer exact match search – an in-house algorithm was used to identify whether an 8 amino acid peptide match existed between the query sequences and sequences within the allergen database. Only matches of 100% similarity over 8 amino acids were considered.

The alignment of the 10 putative peptides present in the 5′ and 3′ insert-flank junctions with database sequences did not identify any matches. Alignment of the six translated insert reading frames with the database sequences resulted in a single 8-mer match between a putative peptide encoded by one of the reading frames in the KWS20-1 insert DNA to a protein in the allergen database (AEV41413.1 – beta-1,3-glucanase from *Hevea brasiliensis* (Pará rubber tree)). However, there is no evidence to suggest that this putative peptide is produced *in planta*, given that it is not in one of the reading frames encoding DMO, PAT or CP4 EPSPS.

Given these results, the risk of allergenic proteins with relevance to human safety being produced by novel RFs generated in KWS20-1 is negligible.

3.4.5.2 Bioinformatic analysis for potential toxicity

The six translated insert reading frames and the putative peptides encoded by the insert-flank junctions were also compared *in silico* to a toxin protein database (TOX_2022). This database is a subset of sequences derived from the Swiss-Prot protein database[10], filtered to remove likely non-toxin proteins, and contained 8,131 sequences at the date of analysis. A FASTA algorithm was used with a BLOSUM50 scoring matrix and the E-value threshold set to 1×10^{-5}. No matches were found between the 10 putative junction peptides and any known protein toxins. Similarly, no matches were identified between the putative peptides encoded by the 6 reading frames in the KWS20-1 insert and any known protein toxins.

The novel RFs in KWS20-1 therefore do not present a toxicity concern.

3.5 Conclusion

Sugar beet line KWS20-1 contains a single copy of the intended DNA insertion, integrated at a single locus in the sugar beet genome. DNA sequencing and Southern blot analysis confirmed that the *dmo*, *pat* and *cp4 epsps* cassettes are present in the KWS20-1 genome with the expected sequence and organisation. No backbone sequences from the transformation plasmid PV-

[8] COMPARE – http://comparedatabase.org/database/
[9] NCBI protein database – https://www.ncbi.nlm.nih.gov/protein/
[10] UniProt – https://www.uniprot.org/

BVHT527462 are present. The inserted DNA is stably inherited and expressed across several breeding generations of KWS20-1. Bioinformatic analyses of the new RFs created by the insertion did not raise any allergenicity or toxicity concerns.

4 Characterisation and safety assessment of novel substances

In considering the safety of novel proteins it is important to understand that a large and diverse range of proteins are ingested as part of the normal human diet without any adverse effects. Only a small number of dietary proteins have the potential to impair health, because of anti-nutrient properties or triggering of allergies in some consumers (Delaney et al. 2008). As proteins perform a wide variety of functions, different possible effects have to be considered during the safety assessment including potential toxic, allergenic or anti-nutrient effects.

To effectively identify any potential hazards, knowledge of the characteristics, concentration and localisation of all newly expressed proteins in the organism as well as a detailed understanding of their biochemical function and phenotypic effects is required. It is also important to determine if the newly expressed protein is expressed in the plant as expected, including whether any post-translational modifications have occurred.

In considering the safety of newly expressed substances, it is important to note that a large and diverse range of proteins are ingested as part of the normal human diet without any adverse effects.

Three novel proteins are expressed in KWS20-1, each of which confer herbicide tolerance: DMO, which confers tolerance to dicamba; PAT, which confers tolerance to glufosinate; and CP4 EPSPS, which confers tolerance to glyphosate.

4.1 DMO

Tolerance to dicamba in KWS20-1 is conferred by the expression of the enzyme dicamba mono-oxygenase (DMO). Wildtype DMO was initially purified from the *S. maltophilia* strain DI-6, which was isolated from soil at a dicamba manufacturing plant (Krueger et al. 1989). DMO prevents the build-up of toxic levels of dicamba (3,6-dichloro-2-methoxy benzoic acid) by catalysing its demethylation to form the non-herbicidal compound 3,6-dichlorosalicylic acid (DCSA) (Behrens et al. 2007).

In *S. maltophilia*, DMO is the final component of a three-component enzyme system, in which electrons move from NADH through a reductase and ferredoxin, before being passed to DMO. Ferredoxin in plant chloroplasts closely resembles the ferredoxin component of this bacterial enzyme system (Behrens et al. 2007). Therefore, DMO in transgenic plants typically includes an N-terminal chloroplast transit peptide (CTP) to allow targeting of DMO to the chloroplast and co-localisation with reduced ferredoxin as an electron source, without the need for the bacterial reductase component (Behrens et al. 2007).

In KWS20-1, the *dmo* expression cassette encodes a precursor protein of 424 amino acids: 340 amino acids encoded by the *dmo* gene, and 84 amino acids encoded by the *rbc*S gene from *Pisum sativum* (pea). These 84 additional amino acids consist of 57 amino acids of CTP and the first 27 amino acids of the *P. sativum* small subunit ribulose 1,5-bisphosphate carboxylase. N-terminal sequencing analysis indicates that processing of the KWS20-1-produced DMO precursor protein results in cleavage of the CTP, leaving a 367 amino acid protein comprised of 27 amino acids of the small subunit ribulose 1,5-bisphosphate carboxylase fused to the N-terminus of DMO. This isoform of the DMO protein, termed DMO+27.1, has an apparent molecular weight of 38.3 kilodalton (kDa).

As well as the additional 27 residual amino acids at the N-terminus, the DMO protein expressed in KWS20-1 differs from the wildtype *S. maltophilia* DMO by an additional leucine at position 2 (Herman et al. 2005). A number of alternatively-processed DMO proteins, some of which contained isoforms with parts of the transit peptide remaining at the N-terminus, have been previously assessed by FSANZ in the following applications:

- A1063 (MON87708 soybean; FSANZ 2012)
- A1080 (MON88701 cotton; FSANZ 2013)
- A1118 (MON87419 corn; FSANZ 2016)
- A1192 (MON87429 corn; FSANZ 2020)
- A1216 (MON94100 canola; FSANZ 2021)
- A1276 (MON94313 soybean; FSANZ 2024)

A comparison of the alternatively-processed DMO proteins in these different plant lines with the wildtype bacterial DMO protein and the DMO expressed in KWS20-1 is shown in Figure 3. The alignment of the amino acid sequences of the different DMO proteins shows they are highly similar. The small differences in sequence are not expected to result in changes in overall structure, immunoreactivity, enzyme activity or substrate specificity (D'Ordine et al. 2009; Dumitru et al. 2009). Each DMO protein has the same function and catalyses the same enzymatic reaction.

As can be observed in Figure 3, the sequence of the DMO protein expressed in KWS20-1 (not including the additional residual RbcS sequence) is identical to those expressed in MON88701 cotton, MON87419 corn, MON87429 corn and MON94313 soybean. In addition, the residual 27 amino acids derived from the *rbcS* gene that are present in the KWS20-1-expressed DMO+27.1 protein are identical to those found at the N-termini of the DMO proteins expressed in MON87708 soybean and MON94100 canola.

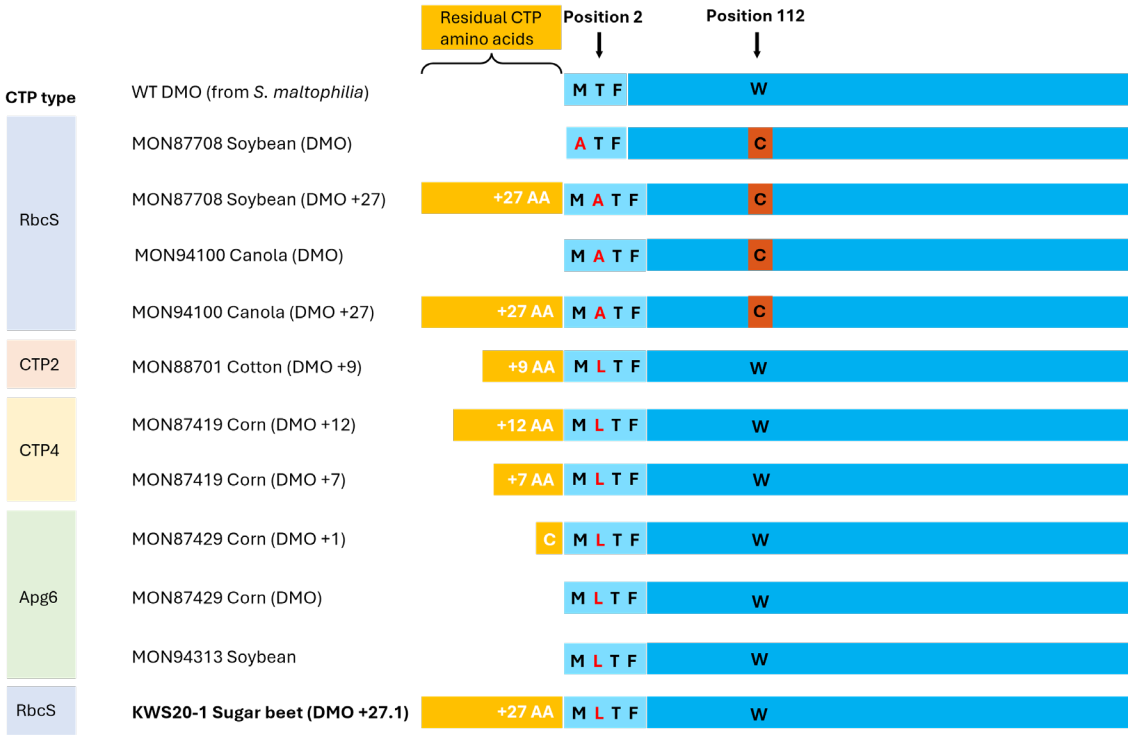

Figure 3. *Forms of DMO protein expressed in different GM commodities compared with wildtype DMO derived from S. maltophilia. Red text/boxes denote amino acid (aa) differences from wildtype DMO. Blue regions indicate areas of 100% amino acid identity. Some GM commodities contain a mixture of alternatively processed DMO proteins. The coloured boxes on the left indicate the origin of the chloroplast targeting peptide (CTP) used for each DMO.*

4.1.1 Safety of the introduced DMO

The DMO protein has been considered in 6 previous FSANZ safety assessments.[11] The detailed safety assessment reports for each of these applications are available on the FSANZ website.[12] In each of these previous assessments, studies on potential allergenicity and toxicity were submitted and assessed. These previous assessments did not raise any safety concerns. Results in the published literature also support the safety of DMO (EFSA 2011; Delaney et al. 2008; Behrens et al. 2007; Chakraborty et al. 2005; Duke 2005; Schmidt & Shaw 2001).

While the sequence of the DMO+27.1 protein expressed in KWS20-1 is not identical in its entirety to any of the previous DMO sequences assessed by FSANZ, Figure 3 shows that the variations to the wildtype protein found in DMO+27.1 (the 27 residual CTP amino acids and the additional leucine at position 2) have all been assessed in previous applications. FSANZ expects that the conclusions of the *in vitro* analyses in these previous assessments would also apply to the DMO+27.1 protein expressed in KWS20-1.

The applicant has submitted further studies with this application which confirm that the DMO+27.1 protein is heat labile and susceptible to pepsin and pancreatin digestion. The heat susceptibility studies used *E. coli*-produced DMO+27.1 as a surrogate for the KWS20-1-produced protein. The equivalence of the bacterially expressed and plant expressed proteins was confirmed by a range of characterisation studies[13]. The applicant also submitted updated bioinformatic studies for DMO that looked for amino acid sequence similarity to known protein allergens and toxins (October 2022). FSANZ has assessed the data submitted by the applicant and the results do not alter conclusions reached in previous assessments.

4.1.2 Conclusion

The DMO expressed in KWS20-1, DMO+27.1, is highly similar to DMO proteins previously assessed by FSANZ. Bioinformatic analyses confirmed that DMO+27.1 has no amino acid sequence similarity to known toxins or allergens. The protein was shown to be heat labile and susceptible to pepsin and pancreatin digestion. Taken together this indicates that the DMO+27.1 protein is unlikely to be toxic or allergenic to humans.

4.2 PAT

The *pat* gene in KWS20-1 encodes the protein phosphinothricin *N*-acetyltransferase (PAT), which enzymatically inhibits phosphinothricin (PPT) (Strauch et al. 1988; Wohlleben et al. 1988). PPT is the active constituent of glufosinate ammonium herbicides and acts by irreversibly inhibiting the endogenous plant enzyme glutamine synthetase. This enzyme is involved in amino acid biosynthesis in plant cells and its inhibition causes accumulation of ammonia, leading to plant death. In glufosinate-tolerant GM plants, the introduced PAT enzyme chemically inactivates PPT by acetylation of the free ammonia group to produce *N*-acetyl glufosinate, allowing plants to continue amino acid biosynthesis in the presence of the herbicide (Hérouet et al. 2005).

The PAT enzyme has been used to confer glufosinate-tolerance in crops for approximately 25 years (ILSI 2016). FSANZ has assessed and approved numerous events with *pat*-encoded glufosinate-tolerance. There have been no credible reports of adverse effects on human health since it was introduced into food.

The wildtype PAT protein encoded by the *pat* gene from *S. viridochromogenes* consists of 183

[11] A1063 – soybean line MON87708 (FSANZ 2012); A1080 – cotton line MON88701 (FSANZ 2013a); A1118 – corn line MON87419 (FSANZ 2016); A1192 – corn line MON87429 (FSANZ 2020); A1216 – canola line MON94100 (FSANZ 2021), A1276 – soybean line MON94313 (FSANZ 2024)

[12] Current status of genetically modified food applications – https://www.foodstandards.gov.au/consumer-information/consumer/current-status-genetically-modified-foods-applications

[13] apparent molecular weight, immunoreactivity, functional activity and glycosylation analysis

amino acids. The PAT protein produced in KWS20-1 is identical to the wildtype *S. viridochromogenes* enzyme except that the N-terminal methionine has been removed co-translationally. This results in a protein comprised of 182 amino acids with an apparent molecular weight of ~22.3 kDa. Identical PAT proteins lacking the N-terminal methionine are found in a number of commercially available glufosinate-tolerant plants.

4.2.1 Safety of the introduced PAT

The PAT protein has been considered in numerous FSANZ safety assessments. These assessments, together with the published literature, have firmly established the safety of PAT and confirm that it does not raise toxicity or food allergenicity concerns in humans (ILSI 2016; Hammond et al. 2011; Delaney et al. 2008; Hérouet et al. 2005).

In previous FSANZ assessments, studies on potential allergenicity and toxicity were submitted and assessed. These previous assessments did not raise any safety concerns and there have been no credible reports of adverse health effects in humans. Since the sequence of the protein expressed in KWS20-1 is identical to the previous PAT sequences assessed by FSANZ, no further safety evaluation is required other than the examination of updated bioinformatics searches.

The applicant has submitted updated bioinformatic studies for PAT that looked for amino acid sequence similarity to known protein allergens and toxins (July 2022). FSANZ has assessed the data submitted by the applicant and the results do not alter conclusions reached in previous assessments.

4.2.2 Conclusion

The data provided by the applicant confirms the PAT expressed in KWS20-1 is identical to previously assessed PAT proteins. Updated bioinformatic analyses confirm that PAT has no similarity with known allergens or toxins that is of significance or concern.

4.3 CP4 EPSPS

The *cp4 epsps* gene in KWS20-1 encodes the protein CP4 EPSPS from the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) family of enzymes, which is ubiquitous in plants and microorganisms. In these organisms, EPSPS catalyses a step of the shikimate pathway, which is responsible for biosynthesis of essential aromatic amino acids and other secondary metabolites (Bentley 1990). Endogenous EPSPS enzymes in plants are inhibited by the herbicide glyphosate (Steinrücken & Amrhein, 1980), which leads to plant death as the plant is deprived of the amino acids and other metabolites needed for normal plant growth and development.

The CP4 EPSPS protein expressed in KWS20-1 is derived from *Agrobacterium* sp. strain CP4, and like other bacterial EPSPS enzymes, has a reduced affinity for glyphosate (Barry et al. 2001; Padgette et al. 1996). In glyphosate-tolerant GM plants into which CP4 EPSPS has been introduced, this reduced affinity allows plants to continue the shikimate pathway and amino acid biosynthesis in the presence of glyphosate.

The CP4 EPSPS protein has been used to confer glyphosate-tolerance in crops for almost 30 years (Green 2009). FSANZ has assessed and approved numerous events with *cp4 epsps*-encoded glyphosate-tolerance. There have been no credible reports of adverse effects on human health since the protein was introduced into food.

CP4 EPSPS encoded by the *cp4 epsps* gene from *Agrobacterium* sp. strain CP4 is a 455 amino acid, 47.6 kDa protein. The CP4 EPSPS protein produced in KWS20-1 is identical to the bacterial CP4 EPSPS protein, which in turn is identical to CP4 EPSPS expressed in a wide range of commercially available glyphosate-tolerant crops.

In KWS20-1, the *cp4 epsps* expression cassette encodes a 531 amino acid precursor protein, consisting of the 455 amino acid CP4 EPSPS protein and a 76 amino acid chloroplast transit peptide (CTP) which targets the CP4 EPSPS protein into chloroplasts. After complete cleavage of the CTP, the mature protein has an apparent molecular weight of ~43.5 kDa.

4.2.1 Safety of the introduced CP4 EPSPS

The CP4 EPSPS protein has been considered in 16 FSANZ safety assessments[14] and has an extensive history of safe consumption (AFSI 2016). These assessments, together with the published literature, firmly establish the safety of CP4 EPSPS and confirm that it does not raise toxicity or allergenicity concerns in humans (Delaney et al. 2008; AFSI 2016).

In previous FSANZ assessments, studies on potential allergenicity and toxicity were submitted and assessed. These previous assessments did not raise any safety concerns. Since the sequence of the protein expressed in KSW20-1 is identical to the previous CP4 EPSPS sequences assessed by FSANZ, no further safety evaluation is required other than the examination of updated bioinformatics searches.

The applicant has submitted updated bioinformatic studies for CP4 EPSPS that looked for amino acid sequence similarity to known protein allergens and toxins (May 2022). FSANZ has assessed the data submitted by the applicant and the results do not alter conclusions reached in previous assessments.

4.2.2 Conclusion

The data provided by the applicant confirms the CP4 EPSPS expressed in KWS20-1 is identical to previously assessed CP4 EPSPS proteins. Updated bioinformatic analyses confirm that CP4 EPSPS has no similarity with known allergens or toxins that is of significance or concern.

4.4 Expression levels of novel proteins

For analysis of the expression levels of the DMO, PAT, and CP4 EPSPS proteins in KWS20-1, tissues were collected from four replicate plots at each of five field-trial sites in representative sugar beet-producing regions of the United States during the 2020 growing season.[15] KWS20-1 was treated with dicamba, glufosinate and glyphosate throughout the growing period. Leaf and root tissue samples were collected from each plot at specified growth stages. See Figure 4 for a summary of sugar beet growth stages and the stage at which each tissue type was collected.

[14] A338, A355, A363, A378, A383, A416, A525, A548, A553, A575, A592, A1049, A1066, A1071, A1097, and A1192

[15] Field sites for testing protein expression levels were in the following states – Michigan, Minnesota, Idaho (2 sites), and North Dakota

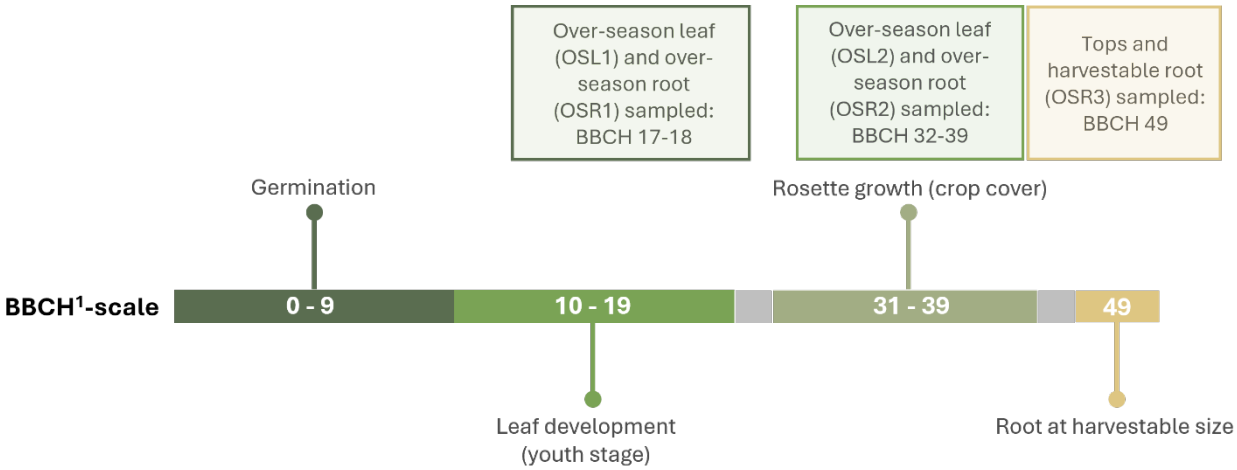

Figure 4. *Growth stages of sugar beet. The stages at which the leaf/tops and root tissues for protein expression analysis were sampled are indicated.*
[1.] BBCH = Biologische Bundesanstalt, Bundessortment und Chemische Industrie – a scale used to identify plant development stages (Meier 2001).

DMO, PAT, and CP4 EPSPS were extracted from tissues using standard methods and their expression levels were quantified in each tissue using a quantitative enzyme-linked immunosorbent assay (ELISA). *E. coli*-derived versions of each protein were used as analytical references for the respective plant-derived proteins.[16]

The mean level of each protein in each tissue type determined by ELISA is shown in Figure 5. Of the three proteins, CP4 EPSPS had the highest expression levels and PAT the lowest expression levels across all tissue types. For all three proteins, the mean expression in herbicide-treated KWS20-1 was highest on a dry weight (dw) basis in Over-season leaf (OSL1) and lowest in harvestable root (OSR3).

For the full set of expression data, including ranges and fresh weight levels, refer to the Application dossier[17] (pages 119 – 122).

4.5 Novel herbicide metabolites in GM herbicide-tolerant plants

FSANZ has assessed the herbicide metabolites for dicamba, glufosinate and glyphosate in GM crops in multiple previous applications. These previous assessments indicate the spraying of KWS20-1 with these herbicides would result in the same metabolites that are produced in non-GM sugar beet sprayed with the same herbicides. As no new herbicide metabolites would be generated in sugar beet event KWS20-1, further assessment is not required.

[16] The applicant has provided data to demonstrate the *E.coli*-expressed versions of these proteins are equivalent to the KWS20-1-expressed proteins and are suitable for use as analytical references.
[17] Application A1303 – https://www.foodstandards.gov.au/food-standards-code/applications/application-a1303-food-derived-herbicide-tolerant-sugar-beet-line

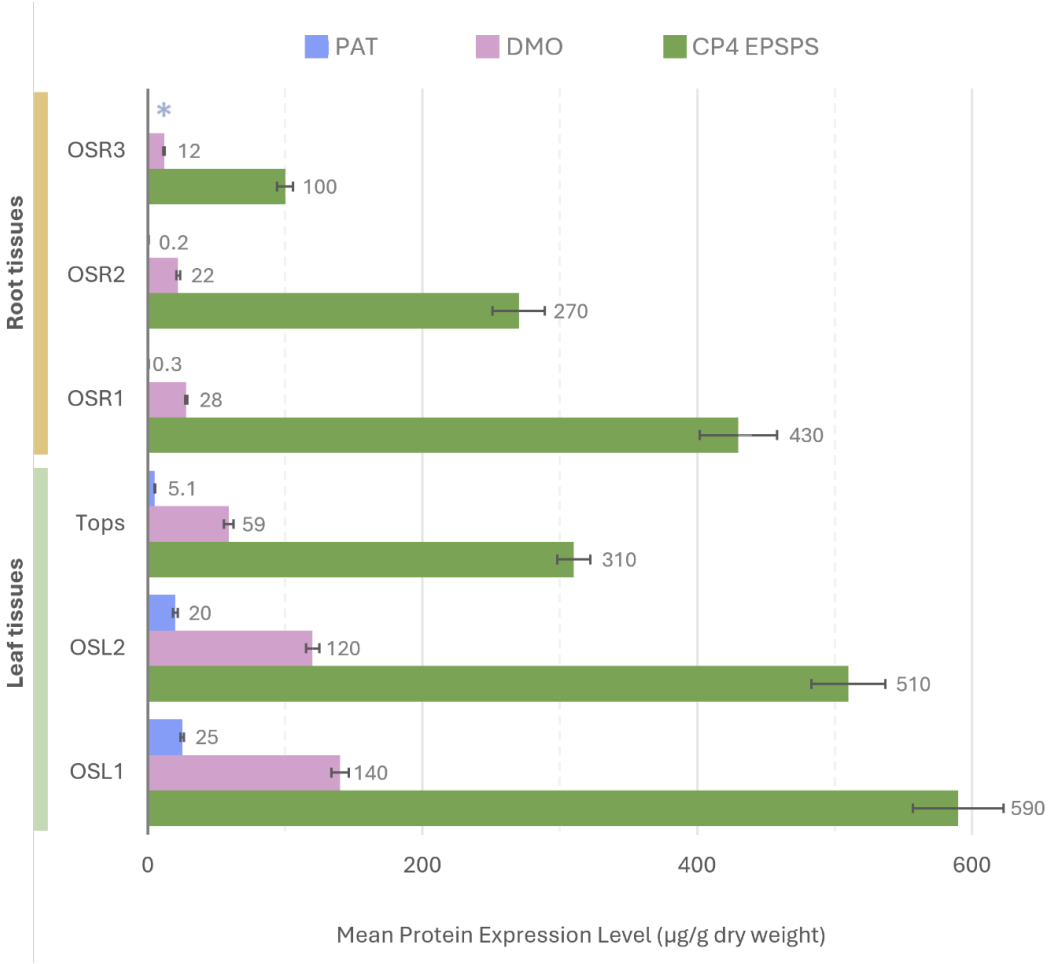

Figure 5. *Mean expression levels of the DMO, PAT and CP4 EPSPS proteins in six tissue types from herbicide-treated KWS20-1. Error bars represent standard error. The asterisk denotes that the level of PAT in harvestable root tissue (OSR3) was below the LOQ (0.125 µg/g dw).*

5 Compositional analysis

The main purpose of compositional analyses is to determine if, as a result of the genetic modification, any unexpected change has occurred to the food. These changes could take the form of alterations in the composition of the plant and its tissues and thus its nutritional adequacy. Compositional analyses can also be important for evaluating the intended effect where there has been a deliberate change to the composition of the food.

The classic approach to the compositional analyses of GM food is a targeted one. Rather than analysing every possible constituent, which would be impractical, the aim is to analyse only those constituents most relevant to the safety of the food or that may have an impact on the whole diet. Important analytes therefore include the key nutrients, toxicants and anti-nutrients for the food in question. The key nutrients and anti-nutrients are those components in a particular food that may have a substantial impact in the overall diet. They may be major constituents (fats, proteins, carbohydrates or enzyme inhibitors such as anti-nutrients) or minor constituents (minerals, vitamins). Key toxicants are those toxicologically significant compounds known to be inherently present in an organism, such as compounds whose toxic potency and level may be significant to health.

5.1 Key components

The key components to be analysed for the comparison of GM and conventional sugar beet are outlined in the OECD Consensus Document on Compositional Considerations for New Varieties of Sugar Beet (OECD 2002). Components assessed in root samples included proximates (protein, total fat, and ash), amino acids, carbohydrates by calculation, sucrose, fibre (crude fibre and pectin), minerals (phosphorus, potassium and sodium) and the secondary metabolite oleanolic acid.

5.2 Study design

KWS20-1 sugar beet and a non-GM control of similar genetic background were grown and harvested from five field trial sites in the United States during the 2020 growing season.[18] The sites were representative of sugar beet growing regions suitable for commercial production. The field sites were established in a randomised complete block design with four replicates per site. Plants were grown under agronomic field conditions typical for each growing region. KWS20-1 plots were treated with dicamba, glufosinate and glyphosate.

Root samples were harvested, ground and stored at -20°C before being shipped to an analytical laboratory on dry ice. Samples were then stored at -20°C until analysis. Compositional analyses were based on internationally recognised procedures including official methods specified by the Association of Official Analytical Chemists (AOAC), the Analytical Oil Chemists' Society (AOCS), methods specified by the manufacturer of the equipment used for analysis, or other published scientific methods.

A total of 29 different analytes were measured in root (see Figure 6 for a complete list). In addition, moisture was also measured and used to convert the analyte values from fresh to dry weight, but was not analysed statistically. Analytes were expressed as percent dry weight (% dw), as shown in Figure 7. Of the 29 components measured, one had more than 50% of observations below the limit of quantification (LOQ) (sodium; listed in grey in Figure 6) and was excluded from the statistical analyses, leaving a total of 28 components that were fully analysed.

A linear mixed model analysis of variance was applied on data combined across the five replicated field trial sites. Statistical analyses were performed using SAS 9.4 (SAS Institute, Cary, North Carolina 2012). For each analyte, ‘descriptive statistics’ (mean, standard error (SE), and range) were generated.

In assessing the statistical significance of any difference between KWS20-1 and the conventional control, a *p*-value of 0.05 was used. Any statistically significant differences were evaluated further to assess whether they were likely to be biologically meaningful. The magnitude of differences in mean values between KWS20-1 and the control were determined, and this difference was compared to the variation observed within the conventional control grown at multiple sites during the 2020 growing season and analysed concurrently with the KWS20-1 and control samples described above (control range).

The natural variation observed in the AFSI Crop Composition Database (AFSI 2020) was also considered. The ranges derived from these values account for variability present in non-GM sugar beet varieties due to a wider range of agronomic and environment conditions, as well as different genetic backgrounds (Harrigan et al. 2010).

Key analyte levels (proximates, carbohydrates and fibre) were also analysed in tops samples but the results are not included in this report. It is noted however that, in the combined site analysis, none of the analyte levels in KWS20-1 tops differed significantly from those of the control.

[18] The states in which the five field trial sites were located: Idaho (2 sites), Michigan, Minnesota, and North Dakota.

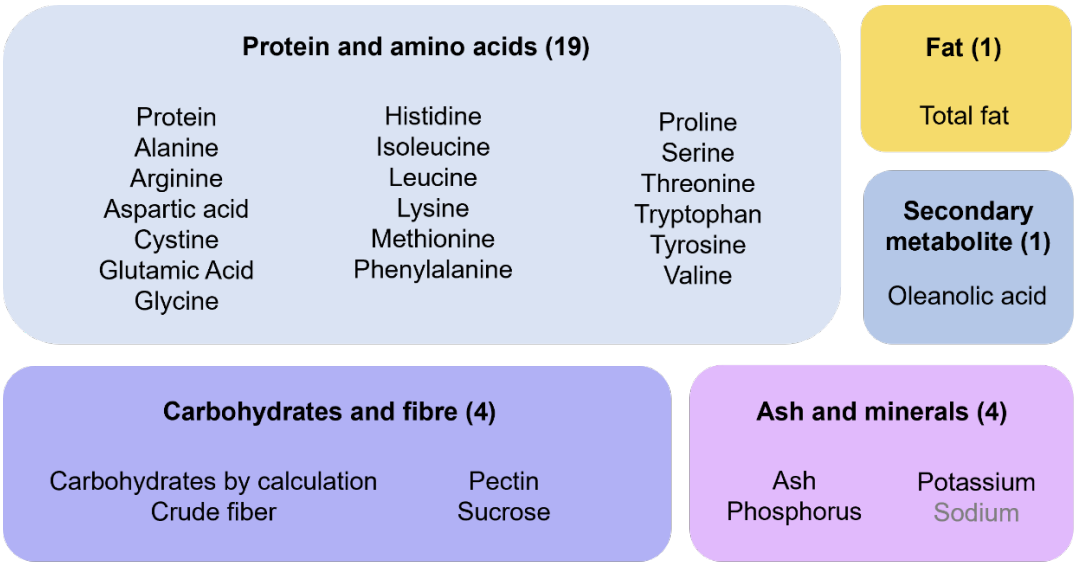

Figure 6. *Analytes measured in KWS20-1 root samples. The analyte listed in grey text had >50% of samples below the LOQ and was excluded from statistical analysis. The analytes listed in black text were analysed fully.*

5.3 Analyses of key components in root

Of the 28 analytes for which mean values were provided, there were 8 for which there was a statistically significant difference ($p < 0.05$) between KWS20-1 and the non-GM control: lysine, proline, serine, threonine, total fat, ash, phosphorus, and potassium. A summary of these 8 analytes is provided in Figure 7. For the complete data set, including values for the analytes for which no statistically significant differences were found, refer to the Application dossier[19] (pages 172-179).

For all analytes where a statistically significant difference was found, except total fat, the deviation of the KWS20-1 mean from the control mean was less than 15% (Figure 7a). The mean total fat in KWS20-1 root was 30.43% higher than that in the conventional control sample. However, as can be observed in Figure 7 (panels b-i), the KWS20-1 mean for all components, including total fat, was within the control range value, indicating that KWS20-1 has a smaller impact on the levels of these analytes than does natural variation within the conventional control. In addition, the observed KWS20-1 means fall well within the natural variability seen in the range of values for conventional reference sugar beet varieties grown in the same growing season (dark grey lines; Figure 7 b-i) and/or the publicly-available AFSI database (light grey bars, Figure 7 b-i). The differences reported here are therefore consistent with the normal biological variability that exists in sugar beet.

Overall, the compositional data are consistent with the conclusion that there are no biologically significant differences in the levels of key constituents in KWS20-1 when compared with conventional non-GM sugar beet varieties already available in agricultural markets. Root from KWS20-1 can therefore be regarded as equivalent in composition to root from conventional non-GM sugar beet.

[19] The Application dossier can be found on the A1303 webpage – https://www.foodstandards.gov.au/food-standards-code/applications/application-a1303-food-derived-herbicide-tolerant-sugar-beet-line

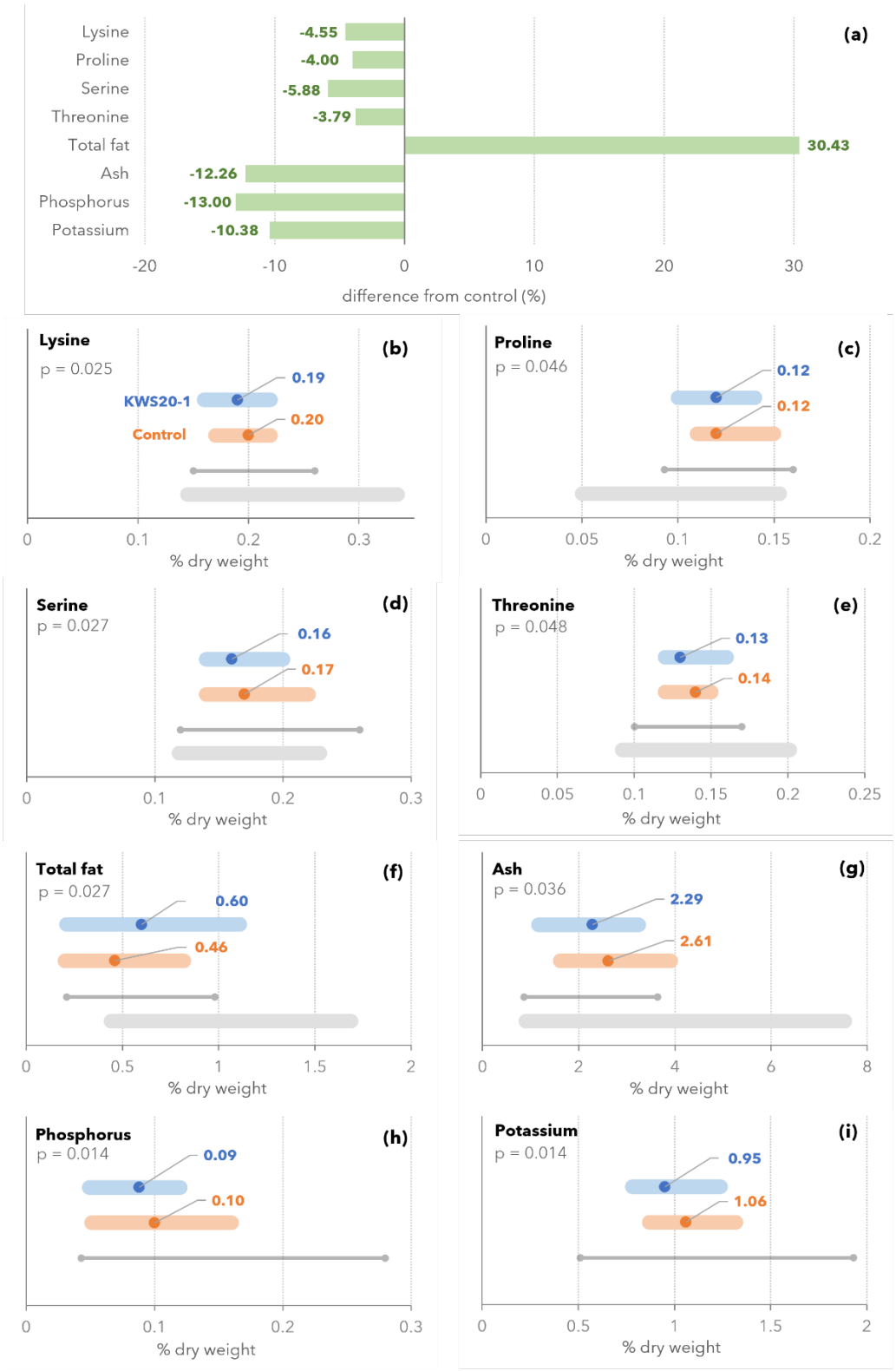

Figure 7. *Visual summary of statistically significant compositional differences between KWS20-1 and the conventional control sugar beet.* **(a)** *Deviation of the mean KWS20-1 value from the mean control value for each of the 8 analytes for which a statistically significant difference was found, expressed as a percentage of the mean control value.* **(b) – (i)** *Measured means (dots) and ranges (coloured bars) for KWS20-1 (blue) and the conventional control (orange) for the 8 analytes as labelled. The pale grey bars represent the range of values for each analyte from the AFSI Crop Composition database (note that values for phosphorus and potassium are not available in the database). The darker grey lines represent the range of values for conventional reference sugar beet varieties grown in the same growing season. Note that the x-axes vary in scale for each component.*

6 Nutritional impact

In assessing the safety of a GM food, a key factor is the need to establish that the food is nutritionally adequate and will support typical growth and wellbeing. In most cases, this can be achieved through a detailed understanding of the genetic modification and its consequences, together with an extensive compositional analysis of the food, such as that presented in Section 5.

Where a GM food has been shown to be compositionally equivalent to conventional cultivars, the evidence to date indicates that feeding studies using target livestock or other animal species will add little to the safety assessment (OECD 2003; Bartholomaeus et al. 2013). If the compositional analysis indicates biologically significant changes, either intended or unintended, to the levels of certain nutrients in the GM food, additional nutritional studies should be undertaken to assess the potential impact of the changes on the whole diet.

KWS20-1 is the result of a genetic modification to confer herbicide tolerance, with no intention to significantly alter nutritional parameters in the food. The compositional analyses have demonstrated that the genetic modification has not altered the nutrient composition of KWS20-1 compared with conventional non-GM sugar beet varieties. The introduction of food derived from KWS20-1 into the food supply is therefore expected to have negligible nutritional impact.

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Appendix 1

PV-BVHT527462-derived genetic elements in T-DNA region

Genetic Element	Location in Plasmid Vector	Description, Source and Reference
Right Border Region	1-357	DNA region from *Agrobacterium tumefaciens* containing the right border sequence used for transfer of the T-DNA (Depicker et al. 1982; Zambryski et al. 1982)
Intervening Sequence	358-401	Sequence used in DNA cloning
***cp4 epsps* cassette**		
***guf-Mt1* terminator**	402-901	3' UTR from an expressed gene of *Medicago truncatula* of unknown function that directs polyadenylation of mRNA (Hunt 1994)
Intervening Sequence	902-907	Sequence used in DNA cloning
***cp4 epsps* coding sequence**	908-2275	Codon optimized coding sequence of the *aroA* gene from the *Agrobacterium* sp. strain CP4 encoding the CP4 EPSPS protein that provides herbicide tolerance (Barry et al. 2001; Padgette et al. 1996)
***CTP2* targeting sequence**	2276-2503	Targeting sequence of the *shkG* gene from *Arabidopsis thaliana* encoding the EPSPS transit peptide region that directs transport of the protein to the chloroplast (Klee et al. 1987; Herrmann, 1995)
Intervening Sequence	2504-2512	Sequence used in DNA cloning
***SAM2-Cm1* promoter**	2513-4516	Intron, 5' UTR, and promoter from a *Cucumis melo SAM2* gene encoding S-adenosyl-L-methionine synthetase which directs transcription (Hernandez-Garcia and Finer 2014)
Intervening Sequence	4517-4522	Sequence used in DNA cloning
***dmo* cassette**		
***DaMV-1* enhancer**	4523-4854	Enhancer from a Dalia Mosaic Virus (DaMV) promoter region (Kuluev and Chemeris 2007) that enhances transcription in plant cells
Intervening Sequence	4855-4864	Sequence used in DNA cloning
***Ubq-Cm1* promoter**	4865-7475	Promoter, leader and intron for a putative ubiquitin protein gene from *Cucumis melo* which directs and regulates transcription (Hernandez-Garcia and Finer 2014)
Intervening Sequence	7476-7486	Sequence used in DNA cloning
***RbcS (Ps)* targeting sequence**	7487-7738	Targeting sequence and the first 27 amino acids from *Pisum sativum* (pea) *rbcS* gene family encoding the small subunit ribulose 1.5 bisphosphate carboxylase protein that is expressed in the chloroplast (Fluhr et al. 1986)
***dmo* coding sequence**	7739-8761	Codon optimized coding sequence for the dicamba mono-oxygenase (DMO) protein of *Stenotrophomonas maltophilia* that confers dicamba resistance (Wang et al. 1997; Herman et al. 2005)
Intervening Sequence	8762-8767	Sequence used in DNA cloning
***guf-Mt2* terminator**	8768-9267	3' UTR from an expressed gene of *Medicago truncatula* of unknown function that directs polyadenylation of mRNA (Hunt 1994)
Intervening Sequence	9268-9273	Sequence used in DNA cloning

***pat* cassette**		
***Cab-At1* promoter**	9274-10661	Promoter and leader from an *Arabidopsis thaliana* chlorophyll a/b-binding (CAB) protein that is involved in regulating gene expression (Ha and An 1988)
Intervening Sequence	10662-10667	Sequence used in DNA cloning
***pat* coding sequence**	10668-11219	Codon optimized coding sequence from *Streptomyces viridochromogenes* for the phosphinothricin N-acetyltransferase (PAT) protein that confers tolerance to glufosinate (Wehrmann et al. 1996; Wohlleben et al. 1988)
Intervening Sequence	11220-11227	Sequence used in DNA cloning
***Hsp20-Mt1* terminator**	11228-11727	3' UTR sequence from *Medicago truncatula* (barrel medic) of a putative *Hsp20* gene encoding a heat shock protein that directs polyadenylation of the mRNA (Hunt 1994)
Intervening Sequence	11728-11779	Sequence used in DNA cloning
Left Border Region	11780-12221	DNA region from *Agrobacterium tumefaciens* containing the left border sequence used for transfer of the T–DNA (Barker et al.1983)

Appendix 2

Development of KWS20-1